VIROLOGY 249, 108–118 (1998)
ARTICLE NO. VY989311

Biochemical and Kinetic Analyses of NS5B RNA-Dependent RNA Polymerase of the Hepatitis C Virus

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Received May 8, 1998; returned to author for revision June 11, 1998; accepted June 26, 1998

The biochemical properties of the RNA-dependent RNA polymerase (RdRp) of the hepatitis C virus were analyzed. A hexahistidine affinity-tagged NS5B fusion protein was expressed with recombinant baculoviruses in insect cells and purified to near homogeneity. Enzymatic activity of the purified protein was inhibited by KCl or high concentrations of NaCl and was absolutely dependent on Mg^{2+}, which could be replaced by Mn^{2+}. NS5B was found to be processive and able to copy long heteropolymeric templates with an elongation rate of 150–200 nucleotides/min at 22°C. Kinetic constants were determined for all four nucleoside triphosphates and different templates. In case of a heteropolymeric RNA template corresponding to the last 319 nucleotides of the hepatitis C virus genome, K_m values for UTP, GTP, ATP, and CTP were ~1.0, ~0.5, ~10, and ~0.3 μM, respectively. The profile of several inhibitors of RdRp activity and substrate analogs indicated that the enzyme has a strong preference for ribonucleoside 5′-triphosphates and that it closely resembles $3D^{pol}$ of picornaviruses.

INTRODUCTION

The hepatitis C virus (HCV) is the major causative agent of parenterally transmitted and sporadic non-A, non-B hepatitis cases worldwide (Choo *et al*., 1989; for a review, see Houghton, 1996). It has been classified as a distinct member of the *Flaviviridae* family that also includes the flaviviruses and pestiviruses (Murphy *et al*., 1995). These viruses have in common a single-stranded RNA genome of plus-strand polarity carrying one long open reading frame that is flanked at the 5′ and 3′ ends by noncoding sequences important for translation and RNA replication. In case of HCV, the genome is expressed as a single polyprotein of ~327 kDa, which is cleaved cotranslationally and posttranslationally by host cell signalases and two viral proteinases into mature proteins (for reviews, see Rice, 1996; Bartenschlager, 1997; Clarke, 1997). Their order within the genome is (from the amino to the carboxyl terminus): NH_2–C–E1–E2–p7–NS2–NS3–NS4A–NS4B–NS5A–NS5B–COOH. The structural proteins C–E2 are the major constituents of the virus particle, whereas the nonstructural proteins 2–5B most likely are important for RNA replication.

Due to the lack of efficient cell culture systems and animal models and the low amounts of viral antigens and RNA in infected tissues, knowledge about the mechanisms of HCV replication is poor. Based on analogy to the closely related flaviviruses and pestiviruses, the well studied poliovirus, and some biochemical analyses of HCV NS proteins (Hijikata *et al*., 1993; Selby *et al*., 1993; Santolini *et al*., 1995; Tanji *et al*., 1995), it is assumed that HCV replication occurs in membrane-associated complexes. Within these, the plus-strand RNA genome is copied into a minus-strand, which in turn is used as a template for synthesis of new plus-strand RNA molecules. At least two viral proteins appear to be involved in this process: the NS3 protein, carrying in the amino-terminal domain a serine-type proteinase and in the carboxyl-terminal two thirds, an NTPase/helicase activity (for reviews, see Rice, 1996; Bartenschlager, 1997; Clarke, 1997), and the NS5B RNA-dependent RNA polymerase (RdRp) (Behrens *et al*., 1996; De Francesco *et al*., 1996; Lohmann *et al*., 1997; Yuan *et al*., 1997).

To better understand the mechanism of HCV RNA replication and to develop appropriate *in vitro* systems, we recently initiated a biochemical analysis of NS5B (Lohmann *et al*., 1997). It is a primer-dependent RNA polymerase able to copy a complete HCV genome in the absence of cofactors. NS5B binds to homopolymeric templates with a distinct specificity [polyuridylic acid, poly(U) > polyguanylic acid, poly(G) > polyadenylic acid, poly(A) > polycytidylic acid, poly(C)], and this order correlates inversely with template activity. Thus, poly(C)/oligo(G) and, to a lesser extent, poly(A)/oligo(U) are used in an RdRp assay with highest efficiencies, whereas poly(G)/oligo(C) and poly(U)/oligo(A) are not accepted as primer–templates (Lohmann *et al*., 1997).

The experiments described here provide a more detailed biochemical and kinetic analysis of the NS5B RdRp activity and its dependence on various parameters and

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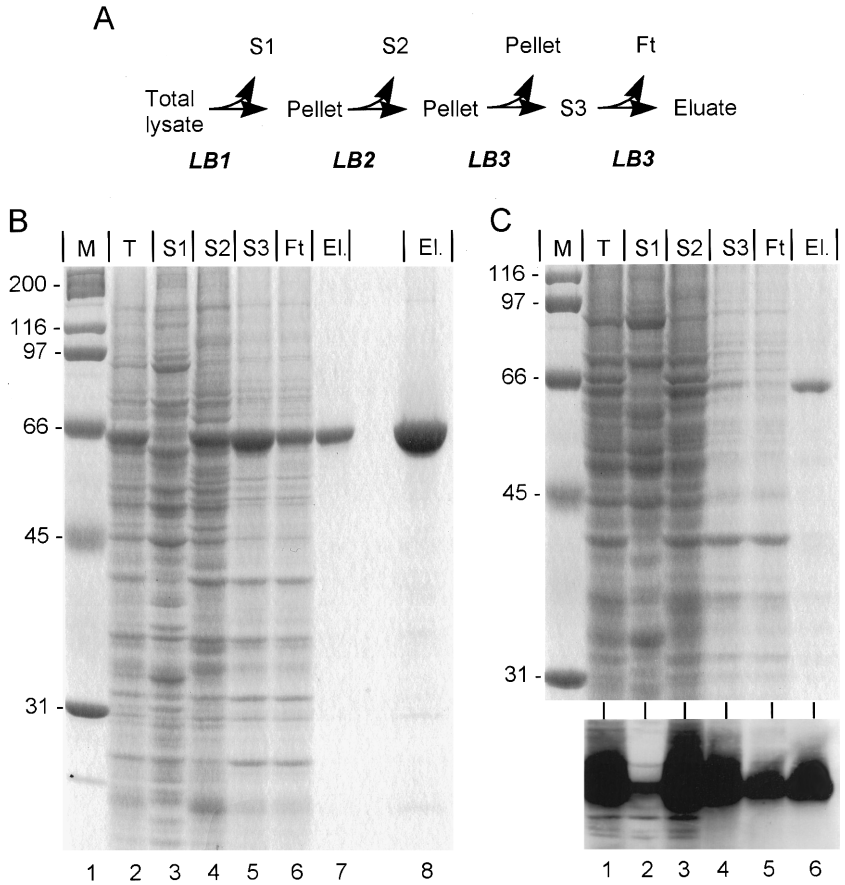

FIG. 1 Purification of NS5B proteins from insect cells. (A) Schematic of the purification method. For details, see the text. Ft, flow through. (B) Purification of $5B^{C\text{-}His}$. One percent of each fraction, corresponding to proteins isolated from 4×10^5 cells, was analyzed by SDS–PAGE and stained with Coomassie Brilliant Blue R-250. (Lane 2) One-half percent of the total cell lysate (T). (C) Purification of $5B^{2\text{-}CHis}$. (Top) Coomassie Blue-stained protein gel loaded with 0.05% of the total cell lysate or 1% of each purification fraction as in B. (Bottom) Result of a Western blot with the same fractions using an NS5B-monospecific antibody. M, Protein molecular weight marker; El., eluate. (Numbers on the left) Sizes of marker proteins (in kDa).

purification procedures. We determined the elongation rate, processivity of the enzyme, and K_m values for all nucleoside triphosphates (NTPs) with different templates. Finally, the influence of various nucleosidic and non-nucleosidic inhibitors on RdRp activity was analyzed.

RESULTS AND DISCUSSION

Purification of HCV polypeptide $5B^{C\text{-}His}$

We recently reported the cloning and expression of HCV polypeptide $5B^{C\text{-}His}$ in insect cells. The protein was tagged carboxyl-terminally with a hexahistidine sequence and expressed with recombinant baculoviruses (Lohmann *et al*., 1997). At 36 h p.i., $5B^{C\text{-}His}$ became detectable as a prominent protein in a total cell lysate and accumulated to high levels 40 h later (Fig. 1B, lane 2). Using subcellular fractionation procedures, we found that in agreement with earlier reports (Behrens *et al*., 1996; Hwang *et al*., 1997), the protein was associated with intracellular membranes (data not shown). Under conditions of high concentrations of salt, detergent, and glycerol, $5B^{C\text{-}His}$ could be solubilized quantitatively, whereas under less stringent conditions, it remained insoluble, which is in contrast to the majority of cellular proteins. We used this property to enrich $5B^{C\text{-}His}$ by successive extractions of cell lysates with buffers with increasing stringencies (Fig. 1A). Cells were lysed in LB1 (see Materials and Methods), and after centrifugation, cellular proteins in the supernatant (S1) were discarded (Fig. 1B, lane 3). After extraction of the pellet and centrifugation, the supernatant (S2) containing essentially cellular proteins and only low amounts of $5B^{C\text{-}His}$ (lane 4) was discarded, and the pellet was extracted with a high stringency buffer (LB3). Under these conditions, the majority of $5B^{C\text{-}His}$ could be solubilized (S3, lane 5) and used for subsequent affinity chromatography with a nickel–nitrilotriacetic acid resin. After several washes, bound proteins were eluted with 250 mM imidazole in LB3 (lane 7). By analyzing 10 μg of the eluted $5B^{C\text{-}His}$ by SDS–PAGE and densitometry scan-

TABLE 1

Enzymatic Activity of $5B^{C\text{-}His}$ RdRp after Dialysis against Various Buffers

Dialysis buffer	Relative activity (%)
	100 ± 4
LB3	91 ± 3
LB3/100 mM NaCl	98 ± 2
LB3/10% glycerol	38 ± 3

Note. Poly(C)-dependent poly(G) reactions were performed in quadruplicate as described at Materials and Methods. The enzymatic activity of the undialyzed $5B^{C\text{-}His}$ protein was set at 100%.

ning of the Coomassie Blue-stained gel, we estimated a purity of >90% (lane 8).

In some experiments, biochemical properties of this $5B^{C\text{-}His}$ protein were compared with those of an NS5B protein (designated $5B^{2\text{-}CHis}$), expressed in the context of an NS2–$5B^{C\text{-}His}$ polyprotein, for two reasons: (1) $5B^{2\text{-}CHis}$ was cleaved intracellularly by the NS3/4A proteinase at the NS5A/B site, releasing an NS5B with an authentic amino terminus (SMS instead of the MAS sequence of $5B^{C\text{-}His}$); and (2) it is assumed that viral RNA replication occurs in membrane-associated complexes containing probably all nonstructural proteins, raising the possibility that they modulate the biochemical properties of NS5B by, for example, influencing its intracellular folding. The expression level of $5B^{2\text{-}CHis}$ was low (Fig. 1C, lane 1). Interestingly, the majority of this protein was enriched in the S2 fraction, and only ~30% was found in S3 (Fig. 1C, lanes 3 and 4). However, to allow a comparison with $5B^{C\text{-}His}$, we sought to purify $5B^{2\text{-}CHis}$ from the same subcellular fraction and under the same conditions. Purity of $5B^{2\text{-}CHis}$ was lower (lane 6), most likely due to binding of cellular proteins to the column matrix, which in the case of $5B^{C\text{-}His}$ were competed by the much higher amounts of this protein.

Dependence of NS5B RdRp activity on reaction conditions

In the first set of experiments, we analyzed whether the high concentrations of salt (NaCl and imidazole) and glycerol used to purify the protein negatively affected enzymatic activity. Therefore, purified $5B^{C\text{-}His}$ was dialyzed against LB3 containing only 10% glycerol or only 100 mM NaCl. As summarized in Table 1, the reduction in the salt concentration had no effect, whereas the decrease in the glycerol concentration down to 10% reduced enzymatic activity to ~38% of the nondialysed control. Therefore, the untreated eluate was used for all subsequent studies.

To determine the optimum concentrations of monovalent and divalent cations for enzymatic activity, poly(C)-dependent poly(G) synthesis was measured under standard conditions (see Materials and Methods) using increasing concentrations of KCl, NaCl, $MgCl_2$, or $MnCl_2$ (Fig. 2). In all cases, the enzyme concentration was adjusted to 100 ng/μl with LB3, and 2 μl was added to a 25 μl reaction, resulting in a final concentration of 40 mM NaCl and 2 mM imidazole in the reaction mixture. Under these conditions, enzymatic activity was highest at low KCl concentrations (0–30 mM) and NaCl concentrations of 80–100 mM (Figs. 2A and 2B). With limiting concentrations of NTPs (0.6 μM GTP), the $MgCl_2$ optimum ranged between 7.5 and 10 mM (Fig. 2C) but increased to 12.5 mM when high NTP concentrations were used in the assay (data not shown). Mn^{2+} could replace Mg^{2+} to about comparable efficiency with slightly lower concentrations required for maximum activity (Fig. 2D). In contrast to some picornaviral polymerases, for which Zn^{2+} appears to be an essential cofactor (Baron *et al*., 1982; Morrow *et al*., 1985), activity of the HCV enzyme was not influenced by this metal ion up to concentrations of 100 μM, whereas higher concentrations led to a partial or complete inhibition (Table 2). Because most NTP preparations are supplied as lithium salts, we also analyzed a possible influence of LiCl on RdRp activity. However, at least under the conditions used here, enzymatic activity was not affected by concentrations of 0.5–10 mM (Table 2). Neutral or slightly acidic pH values were required for optimal activity, and best results were obtained with Tris–HCl or Tris–maleate (Table 2).

Based on these results, we devised two optimized reaction buffers containing 20 mM Tris–HCl, pH 7.0, 12.5 mM $MgCl_2$, and 10 mM KCl or 50 mM NaCl (in either case plus 40 mM NaCl due to the addition of $5B^{C\text{-}His}$ in LB3), both of which provided ~2-fold higher enzymatic activity compared with use of the standard buffer (see Materials and Methods). Therefore, for all subsequent experiments, RdRp assays were performed with an optimized buffer.

Determination of kinetic constants for all four nucleotides

To characterize the enzyme kinetics in greater detail, we first analyzed the time course of RNA polymerase activity using poly(C)/oligo(G) as primer–template. At 22°C, a linear increase in the amount of radiolabeled product RNA was found for incubation times of 10–60 min, whereas during the first 10 min, enzymatic activity was lower (Fig. 3A). Taking time points at closer intervals at the beginning of the RdRp reaction, a lag phase of ~8 min became clearly visible (Fig. 3B), probably corresponding to the time required for the formation of an enzyme–NTP–template preinitiation complex.

Based on these results, we set up experiments to determine the kinetic constants for all four NTPs with different templates. RdRp reactions were performed for 20 min at 22°C using increasing concentrations of one

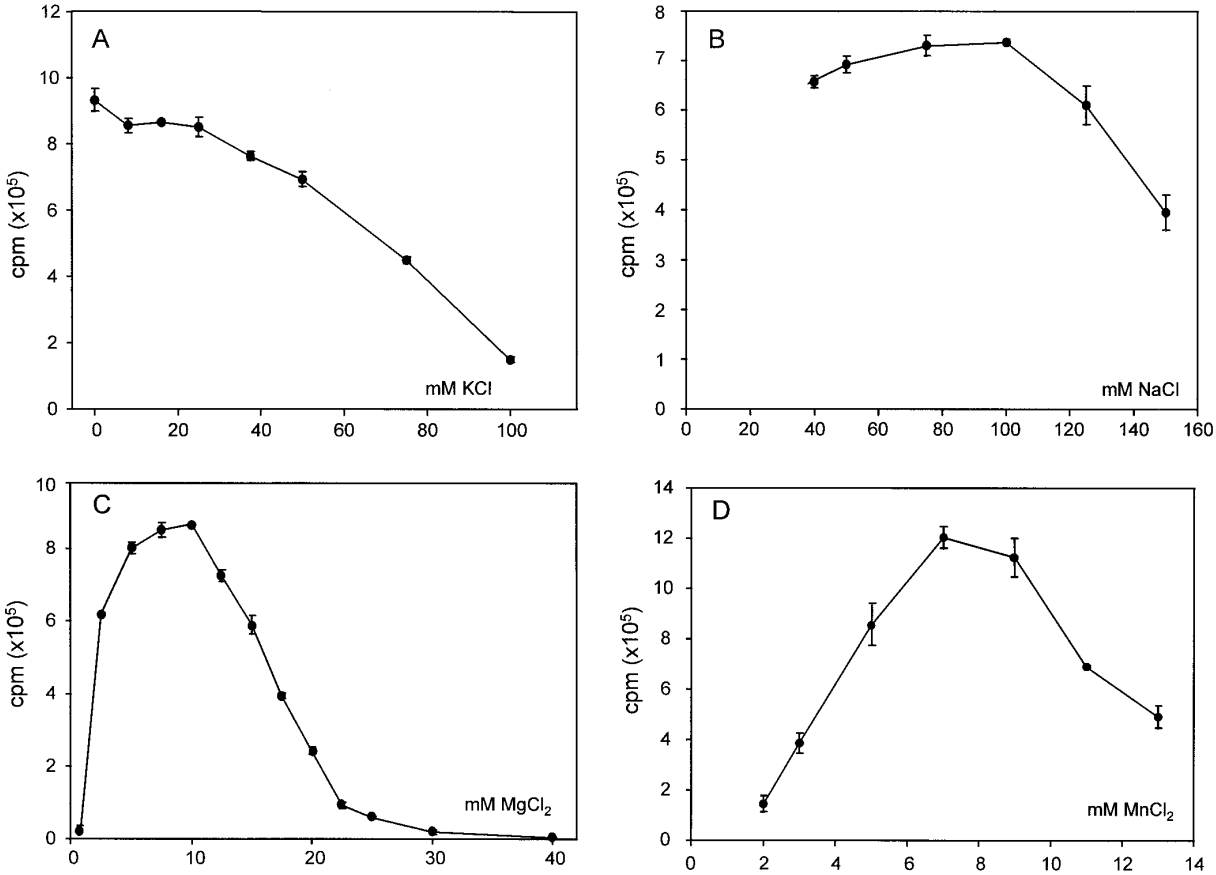

FIG. 2. Influence of monovalent and divalent cations on poly (C)-dependent poly(G) synthesis. Poly(C)-dependent poly(G) reactions were performed in triplicate under standard conditions (see Materials and Methods) using reaction buffers with increasing concentrations of KCl (A), NaCl (B), $MgCl_2$ (C), or $MnCl_2$ (D), and incorporation was determined after TCA precipitation by liquid scintilation counting. (Vertical bars) Range. (A–D) Experiments were not performed in parallel, and therefore the absolute incorporation is not directly comparable.

limiting radiolabeled nucleotide and saturating concentrations of the three nonlabeled NTPs. The results obtained with the heteropolymeric HCV 3′-end RNA are shown in Figure 4, and calculated K_m and V_{max} values are summarized in Table 3. Although K_m values for UTP, CTP, and GTP differed from each other only by a factor of 3, K_m values for ATP were much higher. To exclude the possibility that this high value was due to the template carrying an ~80-nucleotide-long uridine-rich sequence (Tanaka *et al*., 1995; Kolykhalov *et al*., 1996), K_m determination for ATP was repeated with an RNA lacking homopolymeric sequences. Using an *in vitro* transcript corresponding to the *lacZ* gene of *Escherichia coli*, the same K_m value was measured (not shown), indicating that the low affinity for ATP is an NS5B-inherent property.

Because homopolymeric primer–templates can be obtained much easier and in higher amounts, and therefore are frequently used for characterization of enzymatic activities, we also determined the kinetic constants of $5B^{C\text{-}His}$ with homopolymers. Given that the HCV enzyme cannot copy poly(U) and poly(G) (Lohmann *et al*., 1997), K_m values could be determined only with poly(C)/oligo(G) and poly(A)/oligo(U). As summarized in Table 3, much higher concentrations of GTP or UTP were required for optimal enzymatic activity compared with the HCV 3′-end RNA, demonstrating a significant influence of the template on K_m.

Regardless of the templates and reaction conditions used, the apparent enzymatic activity was low (Table 3). For example, when we used poly(C)/oligo(G) as a primer–template, we determined a V_{max} value of 100 pmol of NMP/μg of $5B^{C\text{-}His}$/h, which is 10–20 times lower than the value described for $3D^{pol}$ of poliovirus (Morrow *et al*., 1985; Neufeld *et al*., 1991; Plotch *et al*., 1989). Therefore, we analyzed the influence of the following alterations in the expression and purification procedure on RdRp activity: (1) expression of $5B^{C\text{-}His}$ was carried out at 18°, 22°, and 27°C, and the protein was purified as described above; (2) cells were harvested at various times from 30 to 76 h p.i., and NS5B was purified from S3 under standard conditions; and (3) to exclude the possibility that the NS5B enriched in S3 might represent a poorly soluble subfraction with low enzymatic activity differing in some way from the enzyme soluble under less stringent conditions (S2), $5B^{C\text{-}His}$ was affinity purified from S2 using low or high stringency buffers LB2 and LB3, respectively,

TABLE 2

Effect of Various Reaction Conditions on the RdRp Activity of $5B^{C\text{-}His}$

Parameter	Range tested[a]	Optimum[b]
KCl	0–100 mM	10
NaCl	40–150 mM	80–100
$MgCl_2$	2–50 mM	12.5
$MnCl_2$	2–13 mM	7–9
$ZnCl_2$	0.02–3 mM	[c]
LiCl	0.5–10 mM	[d]
pH	5–8.5	7.0

Note. Poly(C)-dependent poly(G) reactions were performed in triplicates under standard conditions as described under Materials and Methods with given variations of individual parameters.

[a] Range of salt concentrations of pH values used in the RdRp assays as described under Materials and Methods.

[b] Salt concentration or pH value which gave the highest enzymatic activity.

[c] No influence on enzymatic activity at concentrations lower than 100 μM; complete inhibition at concentrations higher than 1.25 mM.

[d] No influence on enzymatic activity within the tested concentration range.

and compared with the protein purified from S3 in the same way. None of these alterations led to a significant increase of enzymatic activity compared with $5B^{C\text{-}His}$ prepared by our standard procedure (data not shown). In all cases, K_m values were virtually constant, whereas V_{max} varied among preparations by a factor of maximally 2, which was the range observed for different $5B^{C\text{-}His}$ preparations purified from S3 under standard conditions.

Several explanations could account for the low apparent enzymatic activity of the purified enzyme. First, NS5B could have a low intrinsic activity *per se*. Second, the enzyme might require an additional cofactor. For example, for poliovirus, it was shown that the RdRp activity of $3D^{pol}$ is stimulated by the VPg precursor 3AB in a concentration-dependent manner (Lama *et al*., 1994; Paul *et al*., 1994), and it seems possible that for HCV, a viral polypeptide exerts an analogous effect on NS5B. Third, only a low proportion of enzyme molecules in our preparation might be active due to, for example, misfolding or aggregation. In agreement with this assumption, preliminary results from gel filtration studies indicate that the purified protein forms high-molecular-weight complexes (V. Lohmann and R. Bartenschlager, unpublished observations). However, so far it is not clear whether these complexes represent preparation artifacts or are a distinct biochemical property of our purified enzyme comparable to the complexes described for poliovirus $3D^{pol}$ (Hansen *et al*., 1997; Pata *et al*., 1995). Fourth, we have recently shown that sequences at the amino terminus of NS5B are crucial for enzymatic activity, whereas alterations at the carboxyl terminus (e.g., a 55-residue deletion) are tolerated (Lohmann *et al*., 1997). To exclude the possibility that the low enzymatic activity was caused by the heterologous sequence at the amino terminus, we expressed a $5B^{C\text{-}His}$ in the context of a larger polyprotein ($NS2–5B^{C\text{-}His}$). This protein was cleaved autocatalytically by the viral proteinases, generating a NS5B protein (designated $5B^{2\text{-}CHis}$) with an authentic amino terminus, which could be purified under standard conditions (Fig. 1C). Using poly(C)/oligo(G) as primer–template, both K_m and V_{max} values were found to be comparable to the values determined for $5B^{C\text{-}His}$ (Table 3), demonstrating that the alterations of the amino-terminal amino acid sequence of $5B^{C\text{-}His}$ had no effect on enzymatic activity.

Elongation rate and processivity of NS5B

As described in the introduction, purified $5B^{C\text{-}His}$ is able to copy a complete *in vitro* transcribed HCV genome in the absence of cofactors. This reaction most likely is initiated by a "copy-back" priming in which 3′-terminal sequences fold back intramolecularly (Behrens *et al*., 1996; Lohmann *et al*., 1997) (Fig. 5A). Under these conditions, the reaction product is a perfectly base-paired

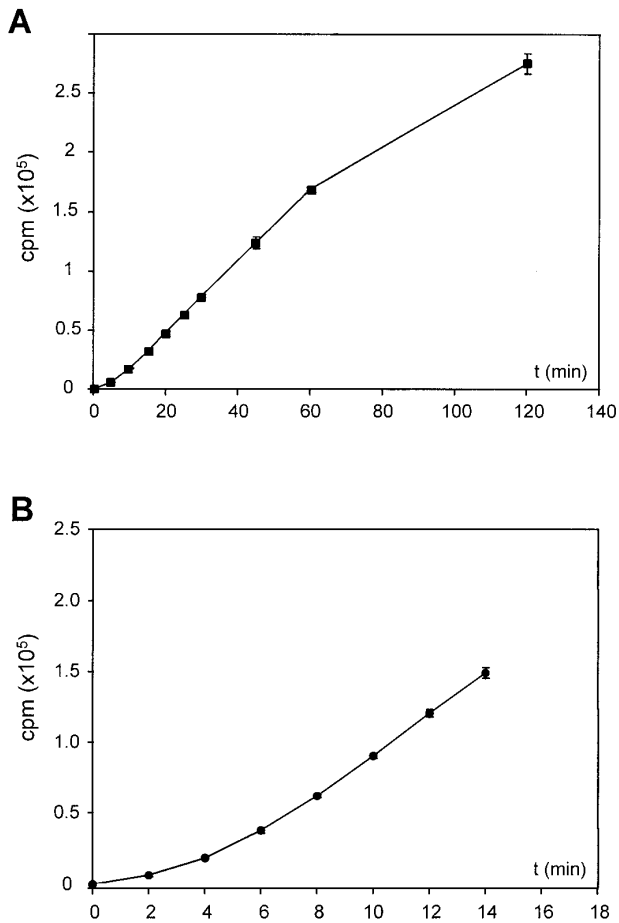

FIG. 3. Time course of RNA polymerase activity. (A) Poly(C)-dependent poly(G) polymerase assays were carried out under standard conditions, and aliquots of the reaction mixture were withdrawn after 5, 10, 15, 20, 25, 30, 45, 60, and 120 min. (B) Time course as in A but with time points taken at closer intervals to show the lag phase.

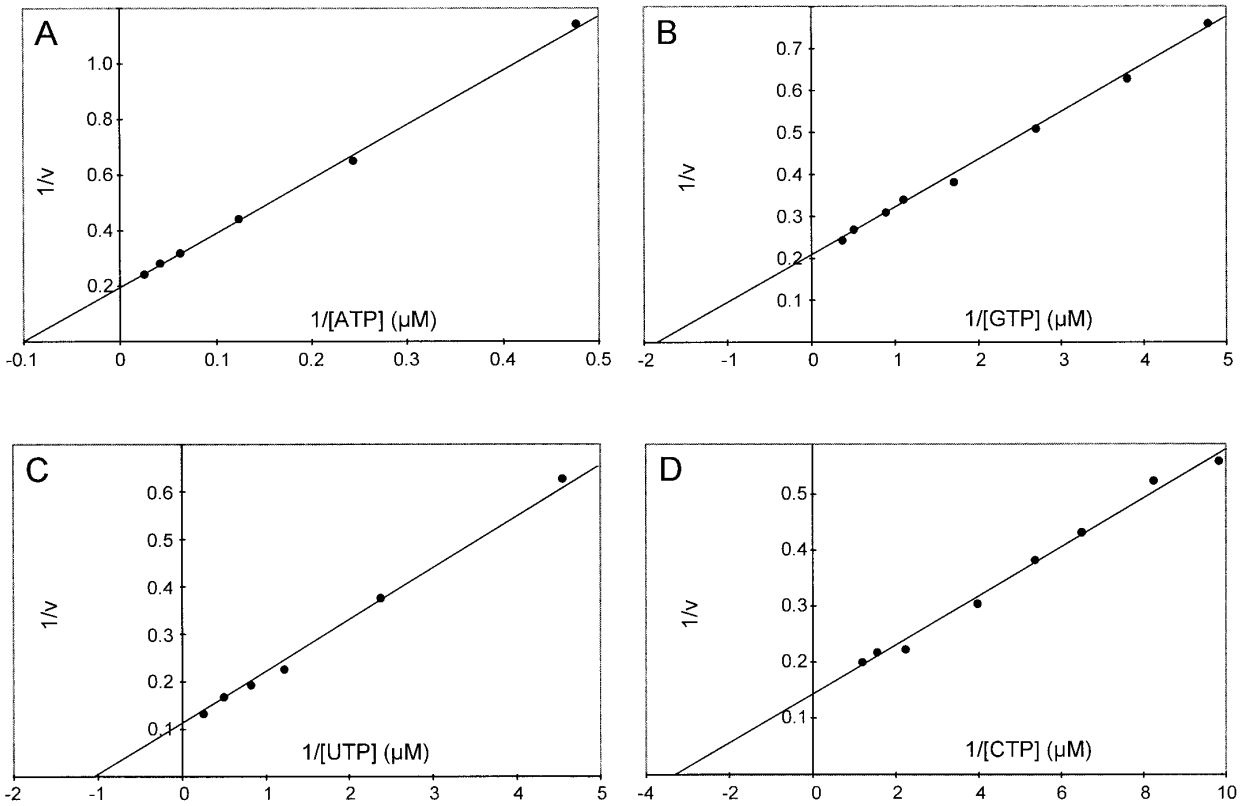

FIG. 4. Effect of varying nucleotide substrate concentrations on RdRp activity. RdRp assays were performed as described in Materials and Methods using the HCV 3′-end heteropolymeric template and the following concentrations of the limiting nucleotide: (A) 2.1, 4.1, 8.1, 16.1, 24.1, and 40.1 μM ATP; (B) 0.22, 0.42, 0.62, 0.82, 1.22, 2.02, and 4.02 μM GTP; (C) 0.22, 0.42, 0.82, 1.22, 2.02, and 4.02 μM UTP; and (D) 0.05, 0.11, 0.21, 0.41, 1.01, and 2.02 μM CTP. Data are represented as double-reciprocal plots (1/v vs 1/[S]). A summary of the K_m values calculated by the program Hyper is given in Table 3.

double-stranded RNA with both strands covalently linked via a single-stranded region. We used this property to determine the elongation rate of the HCV enzyme. An *in vitro* transcribed genomic HCV-RNA was used for an RdRp reaction as described in Materials and Methods. Aliquots of the reaction mixture were withdrawn at regular time intervals, and a portion of the reaction products was analyzed on denaturing formaldehyde-agarose gels. In agreement with the "copy-back" mechanism, the products were larger than the input template (9604 nucleotides; Fig. 5B). However, given the lengths of the products and the difficulty involved in denaturing them completely, they did not allow a careful determination of the elongation rate. Therefore, they were treated with a mix-

TABLE 3

Kinetic Constants of HCV $5B^{C\text{-}His}$ RdRp Activity

Enzyme	Template	Limiting nucleotide	K_m (μM)	V_{max} (pmol of NMP/μg of enzyme/h)
$5B^{C\text{-}His}$	poly(C)/oligo(G)	[α-^{32}P]GTP	2.97 ± 0.42	100
$5B^{C\text{-}His}$	poly(A)/oligo(U)	[α-^{32}P]UTP	22.0 ± 1.7	200
$5B^{C\text{-}His}$	HCV 3′-end	[α-^{32}P]GTP	0.54 ± 0.03	4.8[a]
$5B^{C\text{-}His}$	HCV 3′-end	[α-^{32}P]UTP	0.99 ± 0.1	9.2[a]
$5B^{C\text{-}His}$	HCV 3′-end	[α-^{32}P]ATP	10.08 ± 0.44	5.1[a]
$5B^{C\text{-}His}$	HCV 3′-end	[α-^{32}P]CTP	0.28 ± 0.03	6.7[a]
$5B^{2\text{-}CHis}$	poly(C)/oligo(G)	[α-^{32}P]GTP	2.9 ± 0.4	200

Note. RdRp reactions were performed as described in Materials and Methods with given templates and 500 μM concentration of each nonlabeled nucleotide. K_m and V_{max} values were calculated with the program Hyper.

[a] Values refer to the limiting nucleotide only.

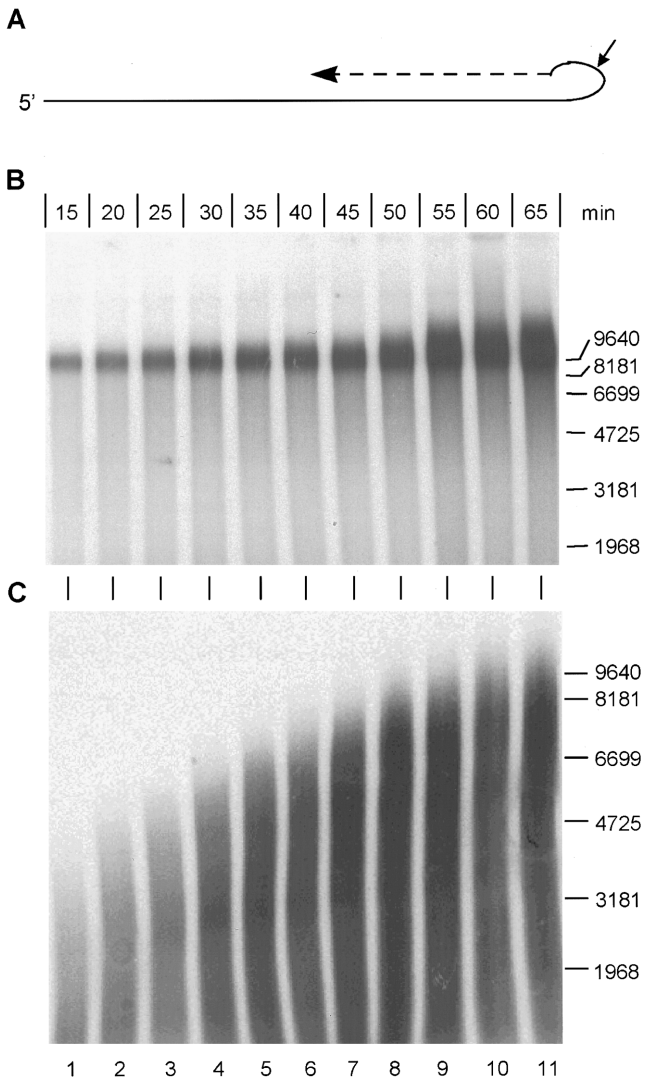

FIG. 5. *In vitro* elongation rate of $5B^{C\text{-}His}$. (A) Schematic of the *in vitro* transcribed full-length HCV RNA template indicating the hairpin structure at the 3′-end used for "copy back" priming. (Arrow) Putative single-stranded region cleaved by RNase under high salt conditions. (B) An RdRp assay was performed with the full-length HCV RNA template, and samples were withdrawn at the indicated times. Reactions were stopped with an SDS-containing buffer, and after isopropanol precipitation, one fifth was analyzed on a denaturing formaldehyde–agarose gel. (C) Fourth fifths of the reaction products were digested with RNases under high salt conditions as described in Materials and Methods and analyzed as in B. (Numbers on the right) Positions of RNA size markers (in nucleotides).

ture of RNases under conditions cleaving only single-stranded sequences. The resulting double-stranded, noncovalently linked RNA products then could be denatured completely and analyzed by electrophoresis in the same way (Fig. 5C). From the linear increase in the size of the RdRp products over time, we calculated an elongation rate of 150–200 nucleotides/min, which is similar to the rates described for the RdRps of poliovirus, encephalomyocarditis virus (EMCV), and rhinovirus (Tuschall *et al*., 1982; Van Dyke *et al*., 1982; Neufeld *et al*., 1991). It should be noted that the exact initiation site on the HCV RNA template is not known, and therefore the elongation rate cannot be deduced from the length of the RNA products generated after the initial 15-min incubation.

Two mechanisms could account for this synthesis rate: (1) the enzyme frequently fell off the template and reinitiated, or (2) the template was copied continuously (i.e., with high processivity). With the first possibility, the elongation rate should depend on protein concentration and be reduced by low amounts of enzyme in the assay, whereas with the second possibility, the elongation rate should be concentration independent. To differentiate between these two possibilities, three RdRp assays were performed as described above using the *in vitro* transcribed HCV RNA template and 200, 50, or 10 ng of $5B^{C\text{-}His}$ (Fig. 6). Regardless of the enzyme concentration, we determined an elongation rate of 150–200 nucleotides/min, indicating that NS5B is processive.

Assuming that the active enzyme is monomeric and contains one active site sufficient for RNA synthesis, we calculated from this elongation rate a theoretical V_{max} value of ~100 nmol of NMP/μg of 5B/h, which is much higher than the "apparent" V_{max} value of 20–40 pmol of NMP/μg of 5B/h [based on an estimation with all four NTPs and the heteropolymeric HCV 3′-end template (Table 3)]. At least two reasons could account for this discrepancy: (1) only a low fraction of our purified NS5B is enzymatically active, or (2) initiation of RNA synthesis is rate limiting. Further studies are required to differentiate between these possibilities.

Sensitivity of $5B^{C\text{-}His}$ to inhibitors of DNA and RNA polymerases and analogs of nucleoside 5′-triphosphates

The RdRp assay reported here also allowed an evaluation of the effects of several nucleosidic and non-nucleosidic agents on enzymatic activity and a comparison with the 3D polymerases of some picornaviruses, in particular the poliovirus, which is described in the literature and in this report. As shown in Table 2, activity of $5B^{C\text{-}His}$ was not stimulated by Zn^{2+}, which is at variance with the results described for poliovirus and rhinovirus $3D^{pol}$ (Baron *et al.,* 1982; Morrow *et al*., 1985). However, analogous to reports on EMCV and poliovirus $3D^{pol}$ (Baron *et al*., 1982; Sankar and Porter, 1992), high concentrations of 1,10-phenanthrolin, a zinc-chelating agent, reduced enzymatic activity of $5B^{C\text{-}His}$ to almost 50% (Fig. 7A), indicating that metal ions are required for NS5B function. Pyrophosphate and the pyrophosphate analogs phosphonoacetic acid and phosphonoformic acid, inhibitors of several DNA-dependent DNA polymerases and reverse transcriptases (e.g., of human immunodeficiency virus), had no significant effect up to concentrations of 1 mM (Fig. 7A).

A moderate inhibition of RdRp activity was obtained

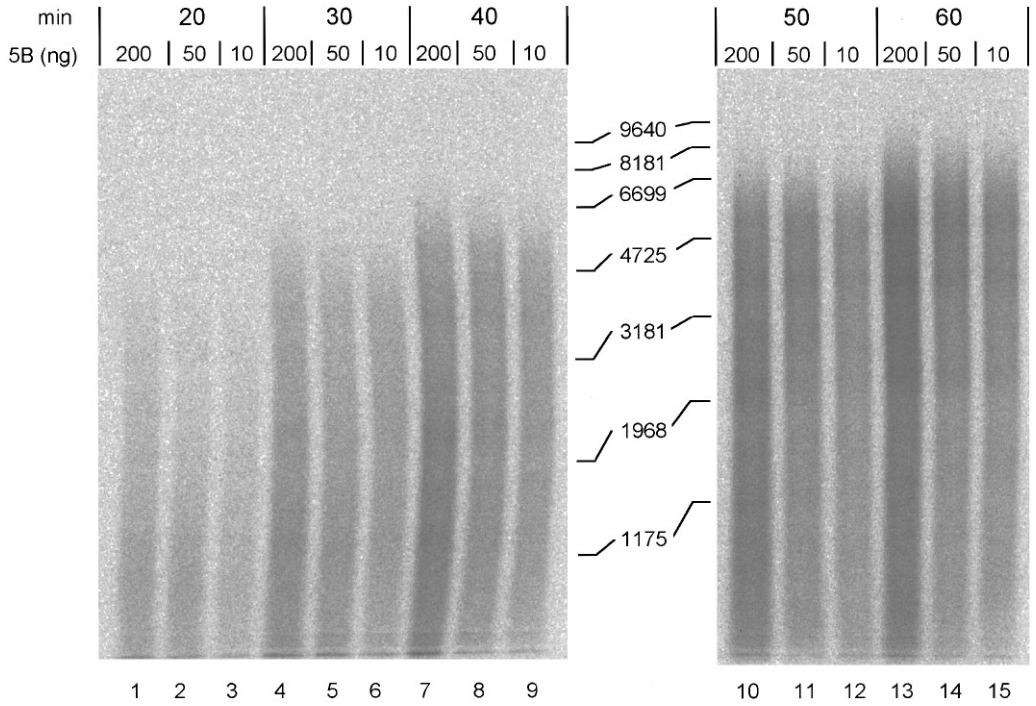

FIG. 6. Processivity of $5B^{C\text{-}His}$. RdRp assays were performed with the full-length HCV RNA, and samples were withdrawn at the indicated times and processed as described in the legend of Figure 5. To correct for the different overall incorporations obtained with the individual enzyme concentrations, 100%, 40%, or 10% of the reaction products was loaded onto the gel for assays performed with 10, 50, or 200 ng, respectively. Radiolabeled RNAs were visualized with a digital phosphorimaging system (BAS2500, Fuji, Tokyo, Japan). (Numbers between the panels) Positions of RNA size markers (in nucleotides).

with cerulenin, a specific inhibitor of fatty acylation and sterol biosynthesis. It was shown that cerulenin blocks poliovirus replication in infected cells (Guinea and Carrasco, 1990) and purified EMCV $3D^{pol}$ *in vitro* with an IC_{50} value of ~1 μM (Sankar and Porter, 1992). Although for HCV NS5B a significant inhibition also was found, the IC_{50} value was ~500-fold higher (Fig. 7A).

It is well known that DNA polymerases and reverse transcriptases are inhibited by substrate analogs functioning as chain terminators; well known examples are acyclovir triphosphate for herpes simplex virus DNA polymerase and azido thymidine triphosphate for human immunodeficiency virus reverse transcriptase. Much less is known about RNA-dependent RNA polymerases. Therefore, we tested the influence of several nucleoside-5′-triphosphates on the enzymatic activity of NS5B (Fig. 7). GDP and GMP reduced enzymatic activity only at high concentrations, indicating a strong preference of the enzyme for the triphosphate group. For dGTP and ddGTP, high concentrations also were required. However, dGTP led to a much stronger reduction, suggesting that the enzyme is more selective toward nucleotides lacking the 3′-OH group than to nucleotides lacking a 2′-OH group. In agreement with this assumption, a nucleoside analog expected to act as a chain terminator for an RNA polymerase (3′-dATP) reduced activity of NS5B to 60% only at a high concentration (10-fold over K_m value; Fig. 7B). A similar result was obtained with poliovirus $3D^{pol}$ (although this enzyme appeared more sensitive), whereas the DNA-dependent RNA polymerase from bacteriophage T7 was readily inhibited by 1 μM 3′-dATP. These results indicate that the HCV enzyme has a strict specificity for ribonucleoside 5′-triphosphates and requires the 2′- and 3′-OH groups.

In summary, the properties described here for NS5B of HCV are very similar to these reported for several picornaviral 3D polymerases (Morrow *et al*., 1985; Neufeld *et al*., 1991; Sankar and Porter, 1992; Jablonski and Morrow, 1995), and they suggest that these enzymes are closely related in function (and possibly structure) and are derived from a common "polymerase module" ancestor (Joyce and Steitz, 1995). Because NS5B is the key player in RNA replication, it might be used as a starting point for the development of an *in vitro* replication system based on the reconstitution of a replicase complex from individual purified components. Furthermore, NS5B is an attractive target for the development of an antiviral therapy. In both cases, a more detailed understanding of the biochemical properties of the enzyme is an absolute prerequisite.

MATERIALS AND METHODS

Materials

All radiolabeled nucleotides (3000 Ci/mmol) were purchased from Amersham Life Science (Braunschweig, Germany). Cordycepin-5′-triphosphate (3′-deoxyade-

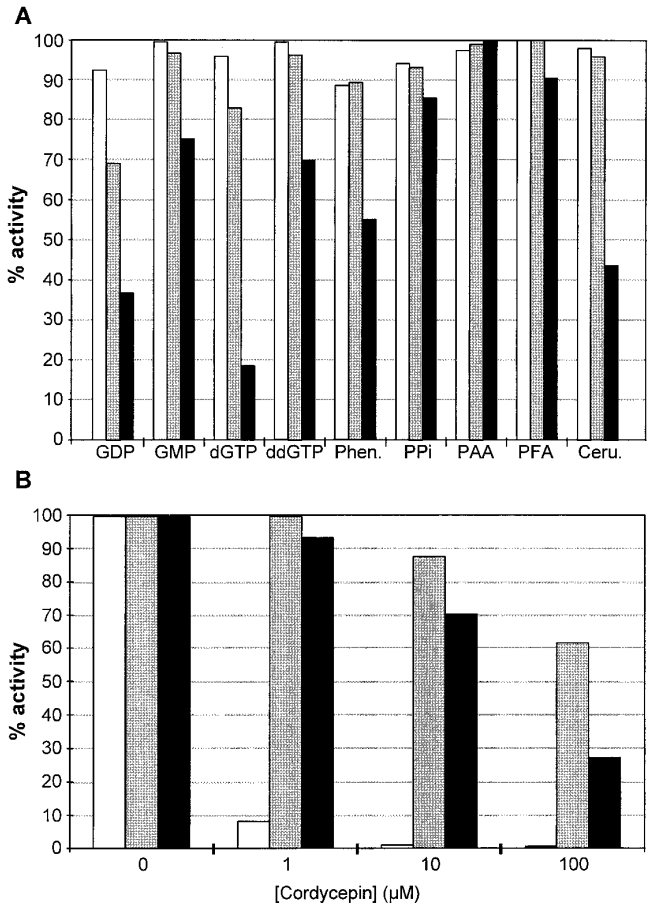

FIG. 7. Effects of inhibitors and substrate analogs on RdRp activity. (A) Poly(C)-dependent poly(G) synthesis was performed as described in Materials and Methods in the presence of 10 μM (□), 100 μM (▨), or 1000 μM (■) GDP, GMP, dGTP, ddGTP, 1,10-phenanthrolin (Phen.), pyrophosphate (PPi), phosphonoacetic acid (PAA), phosphonoformic acid (PFA), or cerulenin (Ceru.). Radioactive incorporation was determined by TCA precipitation and liquid scintilation counting. Values obtained without compounds were set at 100%. (B) RdRp assays were performed with NS5B (▨) or poliovirus $3D^{pol}$ (■) in the absence or increasing concentrations of 3′-deoxyadenosine-5′-triphosphate (cordycepin) using the heteropolymeric HCV 3′-end template. For T7 RNA polymerase (□), the analogous DNA template was used.

nosine-5′-triphosphate), 1,10-phenanthrolin, phosphonoacetic acid, phosphonoformic acid, and cerulenin (2,3-epoxy-4-oxo-7,10-dodecadienamide) were obtained from Sigma (Deisenhofen, Germany). GDP and GMP (disodium salts) were purchased from Sigma–Aldrich (Steinheim, Germany). ddGTP was from Pharmacia (Freiburg, Germany). All unlabeled ribonucleoside triphosphates and dGTP (lithium salts) were obtained from Boehringer-Mannheim Biochemica (Mannheim, Germany) and were of the highest quality available. Homopolymeric RNA templates were purchased from Pharmacia. The 12-mer RNA oligonucleotides were from MWG (Ebersberg, Germany).

Construction of plasmids and generation of recombinant baculoviruses

The basic construct pBac/C-His5B used to express $5B^{C\text{-}His}$ carrying a hexahistidine affinity tag at the carboxyl terminus has been described previously (Lohmann *et al*., 1997). This protein spans residues 2421–3010 of the HCV polyprotein and differs from the authentic NS5B by the presence of two heterologous residues at the amino terminus (MA) and the sequence TSHHHHHH at the carboxyl terminus. To express an NS2–5B polyprotein with a carboxyl-terminal hexahistidine tag, plasmid pBac/C-His2–5B was constructed. A 1952-bp-long *Nco*I/*Sal*I fragment obtained from pTM809–3010 (Bartenschlager *et al*., 1995) and a 4362-bp-long *Sal*I/*Kpn*I fragment isolated after restriction of plasmid pAT1–9604 (Lohmann *et al*., 1997) were inserted into pBac/C-His5B restricted with *Nco*I and *Kpn*I. The resulting plasmid was used to generate a recombinant baculovirus as described recently (Lohmann *et al*., 1997).

Cell culture and protein purification

Expression and purification of NS5B proteins from insect cells were done essentially as described elsewhere (Lohmann *et al*., 1997). In brief, infected cells were scraped off the plate, resuspended in lysis buffer 1 (LB 1) [10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$, and 10 mM 2-mercaptoethanol (2-ME)], and incubated for 30 min on ice. After a 10-min centrifugation at 10,000 *g*, the supernatant (S1) was removed, the pellet was extracted with LB 2 (20 mM Tris–HCl pH 7.5, 300 mM NaCl, 10 mM $MgCl_2$, 0,5% Triton X-100, 20% glycerol, and 10 mM 2-ME), sonicated, and centrifuged. The supernatant (S2) was discarded, and the pellet was extracted with LB 3 (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 10 mM $MgCl_2$, 2% Triton X-100, 10 mM imidazole, 50% glycerol, and 10 mM 2-ME). After centrifugation, proteins contained in the supernatant were applied to a nickel–nitrilotriacetic acid spin column (Quiagen, Hilden, Germany), washed several times, and eluted with 250 mM imidazole and 2 mM EGTA. Purified proteins were quantified using a modification of the method of Lowry (Peterson, 1977). In addition, serial dilutions of the eluate were subjected to SDS–PAGE, and after staining with Coomassie Brilliant Blue R-250, the amounts of NS5B were determined by densitometry scanning and comparison with a dilution series of bovine serum albumin of known concentration analyzed on the same gel. The average concentration of $5B^{C\text{-}His}$ purified with the described method was 0.5–1.0 mg/ml.

Preparation of RNAs for *in vitro* assays

Preparation of the full-length RNA was done as described previously (Lohmann *et al*., 1997) with minor modifications. RNA was transcribed from the linearized

plasmid pAT1–9604 with T7 RNA polymerase, treated with DNase, and after phenol–chloroform extraction and isopropanol precipitation, residual unincorporated nucleotides were removed by gel filtration using Sephadex G-50 NICK columns (Pharmacia). The HCV 3′-end template was prepared in the analogous way using a DNA fragment generated by PCR (Lohmann *et al*., 1997). The transcribed RNA corresponded to the last 319 nucleotides of the HCV genome (9286–9604) and carried at its 5′-end 74 nucleotides of linker sequence. To prepare homopolymeric RNA primer–template mixtures, equal volumes of homopolymer (0.4 μg/μl) and complementary 12-mer primer (4 pmol/μl) were combined, denatured for 2 min at 95°C, and incubated for 5 min at 37°C. Then, 2 μl of this mixture was used for an RdRp assay.

RdRp assays

A standard RdRp assay used to optimize the reaction conditions contained 20 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 25 mM KCl, 0.4 μg of homopolymeric primer–template, 1 μCi of [α-^{32}P]GTP, 0.6 μM nonradioactive GTP, 1 mM dithiothreitol, and 200 ng of purified NS5B (adjusted to 100 ng/μl with LB3 without imidazole) in a total volume of 25 μl. After 2 h at 22°C, the reaction was stopped by the addition of 100 μg of calf thymus DNA and 1 ml of 10% trichloroacetic acid (TCA)–0.5% pyrophosphate (PP_i), and samples were incubated for 30 min on ice. Incorporation of radioactivity was determined by collecting the precipitates on glass microfiber filters (GF/C, Whatman, Kent, England), washing 5 times with 1% TCA/0.1% PP_i, and liquid scintilation counting. Kinetic analyses were done in the same way except that the optimized reaction buffer (20 mM Tris–HCl, pH 7.0, 12.5 mM $MgCl_2$, and 10 mM KCl) and 0.5 μg of heteropolymeric HCV 3′-end template or 0.4 μg homopolymeric primer–template mixture were used. Then, 1–5 μCi of the limiting [α-^{32}P]-labeled NTP, supplemented with varying amounts of the analogous nonlabeled nucleotide, was added, whereas the other three NTPs were present at 500 μM. After 20-min incubation at 22°C, the reaction was stopped by the addition of 100 μl of 100 mM EDTA, and incorporation of radioactivity was determined as described above. For K_m determinations of every NTP–template combination, at least two independent experiments were done in triplicate at six different substrate concentrations of the limiting NTP. RdRp inhibitors were analyzed under standard assay conditions using the optimized reaction buffer of 1 μCi of [α-^{32}P]GTP supplemented with nonradioactive GTP to a final concentration of 8 μM and 500 μM concentration of each remaining nonlabeled NTP. For cordycepin, 5 μCi of [α-^{32}P]ATP, at 16 μM total ATP concentration, and the HCV 3′-end template were used. Reaction conditions for poliovirus $3D^{pol}$ (kindly provided by E. Wimmer and A. Paul) have been described (Cho *et al*., 1993). T7 RNA polymerase was purchased from Promega (Madison, Wisconsin) and used according to the instructions of the manufacturer.

Determination of elongation rate and processivity

Full-length HCV RNA transcripts were used for an RdRp assay under standard conditions with the optimized buffer, 5 μCi of [α-^{32}P]GTP, adjusted to 10 μM with the nonlabeled nucleotide, 500 μM concentration of each remaining NTP, 500 ng of RNA, and varying amounts of purified NS5B. Reactions were stopped by the addition of PK buffer [150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 0.5% (wt/vol) SDS, and 10 μg of tRNA] and incubated with 50 μg of proteinase K for 30 min at 37°C. After phenol–chloroform extraction and isopropanol precipitation, RNAs were dissolved in DEPC-treated water and treated with a mixture of RNase A and RNase T1 under high salt conditions as described recently (Lohmann *et al*., 1997). After proteinase K digestion and isopropanol precipitation, samples were analyzed on 1% formaldehyde–agarose gels.

ACKNOWLEDGMENTS

We are grateful to Ulrike Herian for excellent technical assistance and to E. Wimmer and A. Paul for providing us with purified $3D^{pol}$. This work was supported by grants from the Bundesministerium für Forschung und Technologie (01 KI 96539) and the Deutsche Forschungsgemeinschaft (BA 1505/1–2).

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